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14. ABSTRACT
Cancer mortality in the USA ranks Tennessee number 43 in incidence and 5th in the nation in mortality—both numbers suggesting much work to be done. Altogether, there are over 660,000 citizens with active military or veteran status in our state constituting over 15% of the general population. Therefore, the poor cancer mortality statistics negatively affects not only the state's general population but also military and veteran families living in the state. A self-evaluation of the strengths and weaknesses of our state-wide University of Tennessee Cancer Institute programs revealed two major weaknesses impairing progress toward advancing translational research into the detection and treatment of cancer patients in Tennessee. Both are deficiencies in infrastructure: the first, is the lack of a core diagnostic and prognostic imaging facility and the second, is the lack of a core facility for investigational and therapeutic viral vector production and development. Congress agreed with our self analysis and recognized deficiencies in cancer research infrastructure in our state with its impact on the poor mortality ranking and earmarked funds to bolster our abilities to fight cancer through research into state-of-the-art cancer diagnostics and experimental therapies. In line with the congressional mandate, here we propose to set up two core facilities at the University of Tennessee Cancer Institute: one for the generation of PET biomarkers using microfluidic chemistry and validate their effectiveness for PET/CT-based monitoring of conventional and novel anticancer therapy in animal models and non-small cell lung cancer patients at UT Knoxville; the other a viral vector core for the generation of investigational tools for anticancer therapies at UT Health Science Center Memphis. These cores will work in highly coordinated interactive manner on three interlinked projects.

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Progress Report: W81XWH-07-1-0248 – Year 1
Aim 1. Progress report on preparing ^{18}F-FLT using micro-fluidic chemistry and the visualization of subcutaneous melanoma xenografts in mice at UTGSM

Introduction

This proposal sets the goal to create the infrastructure for delivering and evaluating gene therapy based interventions against cancer. The work is subdivided into tow segments. One part is to develop a core for the synthesis of a new positron emitting (PET) tracer, 3'-Deoxy-3'-[^{18}F]fluorothymidine: ([^{18}F]FLT), to image cancer using microfuidic chemistry. The second part is for the development of a viral vector core (VVC) for the generation of recombinant viruses targeting cancer cells.

The first year of the two year award was spent on setting up the infrastructure for these core laboratories. Both of these cores are now near operation and we anticipate the completion of the proposed proof of principle experiments during the second year of the award.

Key Research Accomplishments

Objective 1: With the support of the DOD grant, we have developed and tested chemistry routes for the synthesis of 3'-Deoxy-3'-[^{18}F]fluorothymidine: ([^{18}F]FLT) using an automated microfluidic platform. In addition, we have evaluated the growth of the murine melanoma B16/F10 tumor in mice and validated the ability to image these tumors by micro positron emission tomography (PET) using [^{18}F]-2-fluoro-2-deoxy-D-glucose.

1. Infrastructure and Personnel

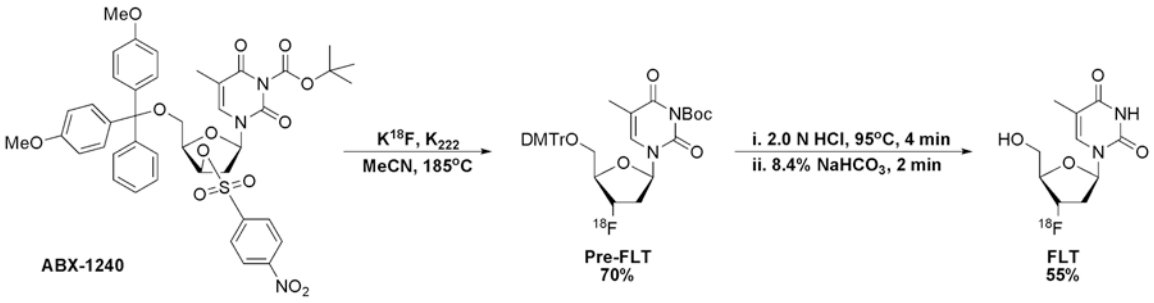
The PET biomarker core (PBC) facility is located in a dedicated PET imaging area which also houses organic chemistry laboratories, the Preclinical and Diagnostic Molecular Imaging Laboratory (PDMIL) and is adjacent to the recently completed PetNet production facility which supplies F-18 to the PBC generated on an RDS 111 (Eclipse HP) negative ion cyclotron with an 11-MeV proton energy. The laboratories within the PBC have recently been remodeled to provide areas dedicated to purification and analysis of biomarkers contiguous with the wet-lab that contains the hot-cell. To perform the synthesis of ^{18}FLT we purchased a Minuteman Liquid Flow Microchemistry platform (Advion Biosystems, Ithaca, NY). To assess the radiopurity of biomarkers generated using the Minuteman the PBC lab also purchased an AR-2000 thin layer chromatography scanner (Bioscan Inc, Washington DC) with WinScan 2D imaging software. A 7890A Gas Chromatography (GC) system (Agilent Technologies, Santa Clara, CA) was also added to the laboratory to analyze the chemical purity of the organic solvents used to generate ^{18}FLT and other biomarkers produced by the PBC.

2. Microfluidic synthesis of ^{18}FLT

3'-Deoxy-3'-[^{18}F]fluorothymidine: ([^{18}F]FLT) is a structural analog of the DNA constituent, thymidine. [^{18}F]FLT is a radiolabeled imaging agent that has been proposed for investigating cellular proliferation with positron emission tomography (PET). Although [^{18}F]FLT is not incorporated into DNA it is trapped in the cell, due to phosphorylation by thymidine kinase, a part of the proliferation pathway. As such it has the potential to image proliferating tumor due to the increase in DNA synthesis rate.

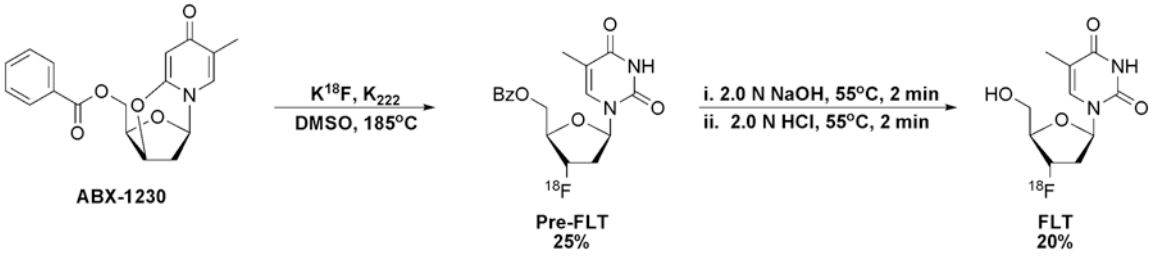
We have investigated two synthetic pathways using the NanoTek LF microfluidic synthesizer. The first involved the displacement of a nitrophenylsulfonyl group at the 3'-hydroxy position (Scheme 1). This labeling occurred in high yield in the microfluidic reactor; however the second phase of the synthesis, hydrolysis, was not nearly as efficient. A modified hydrolysis vessel was constructed and tested to provide [^{18}F]FLT for purification and QC. The overall yield of this process has been on the order of 55% from the start of synthesis.

Scheme 1: Synthesis of [^{18}F]FLT from 3-*N*-Boc-5'-*O*-dimethoxytrityl-3'-*O*-nosyl-thymidine.



Our second approach began with the more common 5'-*O*-Benzoyl-2,3'-anhydrothymidine precursor (Scheme 2). The labeling step was problematic from the beginning. We used an array of solvents and found the best was DMSO, generating a modest 25% yield in the labeling step. This reaction, however, was not reproducible in the microfluidic reactor. The benzoyl deprotection, on the other hand, was a very efficient process using 2.0 N sodium hydroxide.

Scheme 2: Synthesis of [^{18}F]FLT from 5'-*O*-Benzoyl-2,3'-anhydrothymidine.



To aid purification of [^{18}F]FLT we directly connected an HPLC to the NanoTek LF unit (Figure 1). The [^{18}F]FLT final product solution was collected using a Waters® XTerra® column (150 x 3.4 mm) and a 5% ethanol (aq) mobile phase at 2.0 mL/min. The retention time was ~ 8-10 minutes.

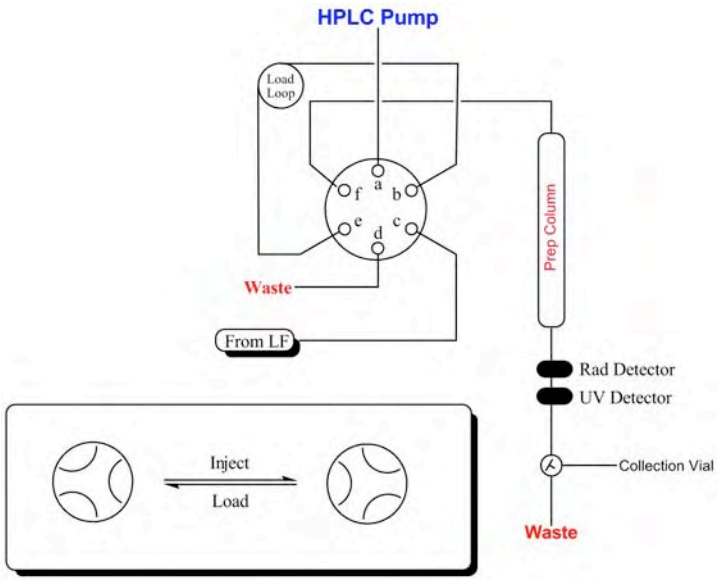


Figure 1: NanoTek LF to HPLC injector loop.

In collaboration with Dr. Giamis and his colleagues at Advion (formerly Nanotek Inc.) we also tested two different sets of automation that control the synthesis of [^{18}F]FLT:- Sequence Discovery (Figure 2) and Batch (Figure 3). The Sequence Discovery mode allows the operator the ability to quickly optimize labeling or hydrolysis reaction conditions. Unique to the Discovery modes is the joining of reagent flows at a "mixing T" prior to the reactor. As the reagents meet, in a laminar flow environment, the residence time and temperature in the reactor generate the products in high yield. The Batch mode involves the pre-mixing of the precursor and the dried [^{18}F]fluoride. The solution is then pumped through the reactor at the determined rate and temperature for optimal yield. This mode of operation will be used primarily for high radioactivity production runs.

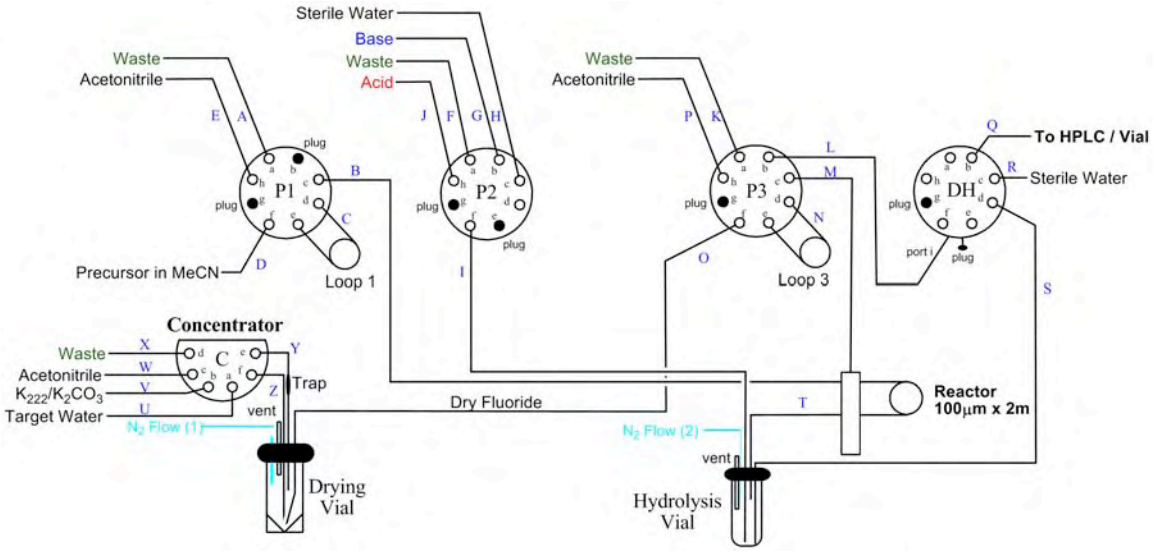


Figure 2: Sequence Discovery plumbing diagram.

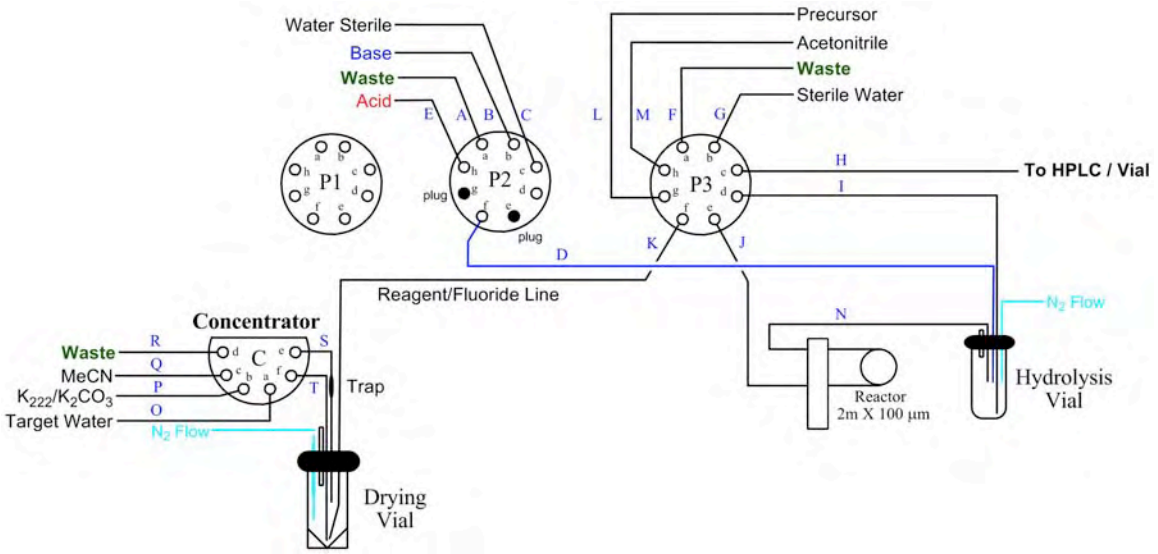


Figure 3: Batch plumbing diagram.

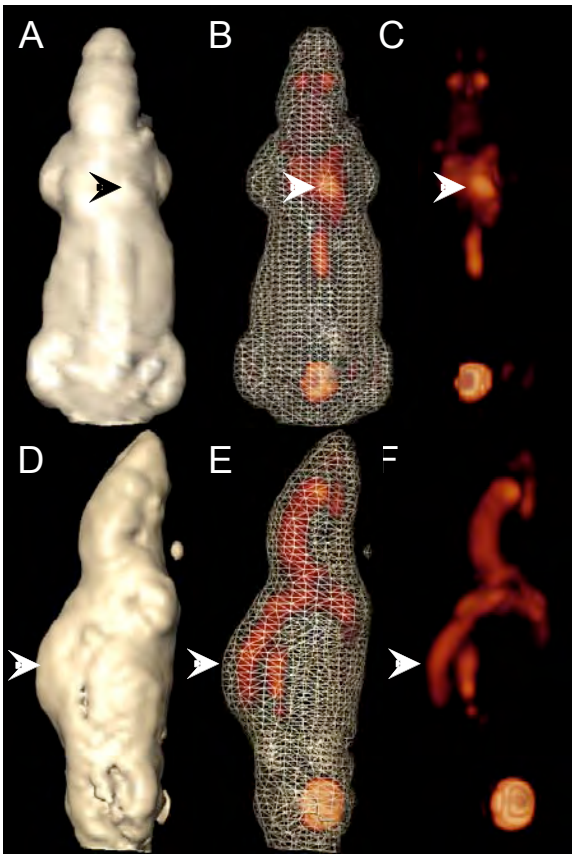


Figure 4. PET imaging of FDG in mice with B16/F10 tumors. ^{18}FDG injected iv was evidenced in the sc B16/F10 xenograft (arrowheads) in live mice using micro PET imaging. A & D) Volumetric representation of the mouse, B & E) Subcutaneous appearance of ^{18}FDG in the tumor, and C & F) ^{18}FDG PET activity image alone.

In the past three months, Advion/Nanotek have normalized the synthesis process, plumbing, and purification and established a set of criteria and standard testing protocols (STP) to be used for the quality control of the [^{18}F]FLT final product. Using these STP's, we have met every qualification for the release of the final product. Our progress to date is limited by the number of successful, complete runs we have performed. Due to our focus on developing the methodology, automation, and technology, we have had little time to carry out multiple repeat syntheses of [^{18}F]FLT. This will not be the case in later parts of the grant application where our focus will be to produce qualified doses of [^{18}F]FLT for use in the murine model of metastatic melanoma.

Live PET imaging of B16/F10 xenografts in mice

A major preliminary goal was to assess the ability of ^{18}FLT to image melanoma xenografts implanted in C57BL/6 mice and to further compare this with the commonly used biomarker ^{18}FDG. Mice were injected with 10^6 B16/F10 murine melanoma cell sc lin C57Bl/6 mice and left for 10 days. After this time, mice received ~300 µCi of ^{18}FDG (purchased from PetNet) and 30 min thereafter the mice were anesthetized using 1-3% isoflurane administered from a

vaporizer in 2 L of oxygen and delivered through a nose-cone. The PET data were acquired using a microPET P4 apparatus (Siemens Preclinical Solutions, Knoxville, TN). The data were reconstructed using a 2D ordered subset expectation maximization (OSEM) algorithm and are visualized using the image analysis software Amira.

The PET images revealed the presence of a subcutaneous lesion indicating uptake of [^{18}F]FDG in the lesions; however there was considerable accumulation within the interscapular brown fat, heart, and abdominal spine. Notably, the lungs were devoid of activity. We will continue these experiments by introducing the B16/F10 cells iv to induce pulmonary lesions. These mice will then be imaged using [^{18}F]FDG in order to test the efficacy of this biomarker at imaging the small lesions that would likely develop in the lungs within 21 days post-injection.

Objective 2. Progress report on establishing a viral vector core (VVC) laboratory at the UTHSC

With the support of DOD grant we have set up the viral vector core laboratory at the UTHSC, started from last July, what we have achieved so far include the following programs.

1. Infrastructure and Personnel

The VVC core laboratory is located in rooms 276, 278 and 279 of the Cancer Research Building at the UTHSC. Room 279 is wet bench laboratory, where we make adenoviral and lentiviral vectors using standard molecular biology techniques. In this fully functional laboratory we have acquired the following equipment to perform our research, such as a PCR thermocycler, three gel electrophoresis units, two power suppliers, two table-top centrifuges, a microwave, two heat blockers, a pH meter, a water bath, a -20°C freezer and a refrigerator.

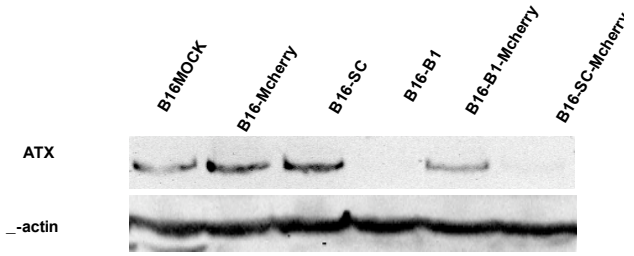


Fig.1 Knockdown ATX in B16 cells using lentiviral shRNA. (B16 SC: scramble shRNA transduced B16 cells; B16-B1: lentiviral shRNA against ATX transduced B16 cells)

In the adjacent rooms 276 and 278, we have established the BL-2 level tissue culture facility. In room 276, which is used for maintaining normal cell line and adeno-associated viral vector work, there is one biosafety cabinet, one ultracentrifuge and two CO2 incubators. In room 278, there are two separate biosafety cabinets, one designed for lentiviral vector production and the other for adenoviral vector production, as well as a table-top centrifuge and one inverted fluorescent microscopy. In the common equipment room 204, we have a -80°C freezer and one cell storage unit.

We have hired three people for this project, one is the director Dr. Junming Yue, who is in charge of the routine operation of this core laboratory. He supervises senior research assistant Dr. Aixia Ren, and research assistant Sravaya Penmatsa.

The VVC laboratory has received approval from the Institutional Biosafety Committee of the UTHSC to produce both lentiviral and adenoviral vectors.

2. Lentiviral Vector system

The lentiviral vectors for gene overexpression and gene knockdown using shRNA or miRNA have been constructed and tested. First we produced autotaxin (ATX) shRNA and scrambled lenitviral vectors. The ATX lenti-shRNA have been transduced into B16 melanoma cells and MDA-231 cells. The

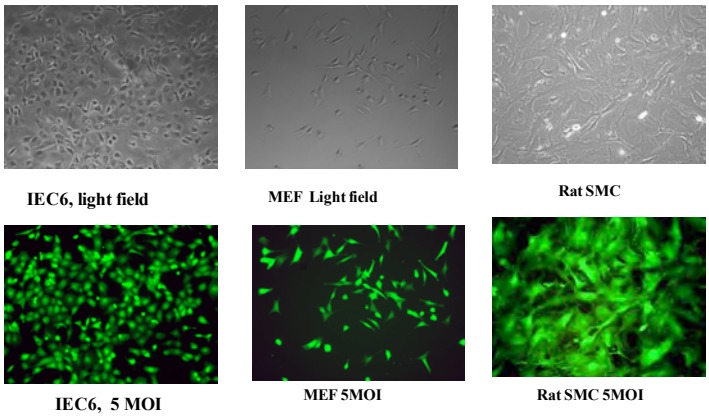


Fig.2 Lentiviral EGFP vector transduced different cells. (IEC6: rat intestinal epithelial cells, MEF: mouse fibroblasts, SMC: rat smooth muscle cells)

endogenous ATX expression was greatly reduced in ATX lenti-shRNA transduced B16 cells compared with the cells transduced with scrambled shRNA (Fig.1). The miRNA mediated ATX knockdown vector has also been generated. In an initial test we observed dramatic knockdown of ATX expression.

In addition, we have constructed EGFP, lysophosphatic acid (LPA) and four more mutant lentiviral vectors. The stable expression cell lines have been established using these vectors. The EGFP lentiviral vector has been used to transduce several different cell lines, such as mouse fibroblasts, rat intestinal epithelial cells, rat smooth muscle cells, which resulted in transduction efficiency of more than 95% in all cell lines exmained (Fig.2).

We also constructed a doxycline induced lentiviral vector system, which showed no background leakiness. We tested this system in MDA-231 cells using EGFP reporter gene, which a robust EGFP expression was induced with the doxcycline at a concentration of 1ug/ml (Fig.3).

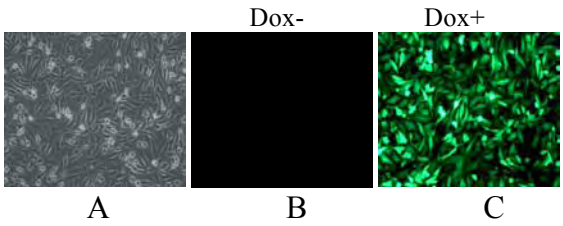


Fig.3 Doxcycline induced EGFP expression in lentivral vector transduced MDA-231 cells. (A: MDA-231 cells under light field, B: no doxcycline, C: with doxycline)

As proposed in this grant, The VVC has constructed a STAT3, a dominant-negative STAT3 (DN-STAT3), as well as two additional mutant STAT3 lentiviral vectors. All four mutant STAT3 constructs have been transduced into mouse fibroblasts with high efficiency (~90%.) By immunoblotting we have determined that transduced cells have high and stable expression of STAT3 (Fig. 4).

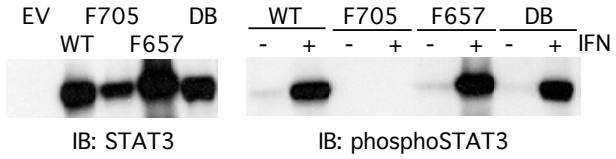


Fig.4 Lentiviral STAT3 vectors transduced into STAT3- mouse embryo fibroblasts. (EV: empty vector; WT: wild type STAT3, F705: STAT3 mutation in tyrosine phosphorylation site, F657: STAT3 mutation in putative PI3K binding site; and DB: STAT3 mutation in putative DNA binding site.). Cells were treated in the absence or presence of interferon (IFN) and immunoblotted with Abs against STAT3 or phospho-STAT3.

3. Adenoviral vector system

The VVC has constructed adenoviral vector systems for gene expression, shRNA-based gene knockdown and doxycline-inducible gene expression and miRNA-mediated gene knockdown systems.

For gene overexpession and shRNA-mediated gene knockdown, the gene of interest can be subcloned into multiple cloning sites and driven by CMV promoter in a shuttle vector with the adeno-Easy system. The polymerase III promoter H1 and U6 promoter has been inserted in a shuttle vector and are ready to express shRNA in the proposed studies to knockdown gene expression.

We have also constructed a single adeno-genome vector for doxycline-inducible expression that the reverse transactivator rtTA3, a third generation production has been inserted in E3 region, and the gene of interest is inserted in E1 region of adenogenome. We have validated the inducibility of this system with a EGFP reporter gene. The replication defective viruses produced can efficiently transduce human HeLa cells (Fig.5) rat smooth muscle cells following the doxycline treatment.

We also established a miR-21 based doxycline gene knockdown adenoviral vector. We found that this system can efficiently knockdown the expression of an EGFP reporter after induction with doxycline.

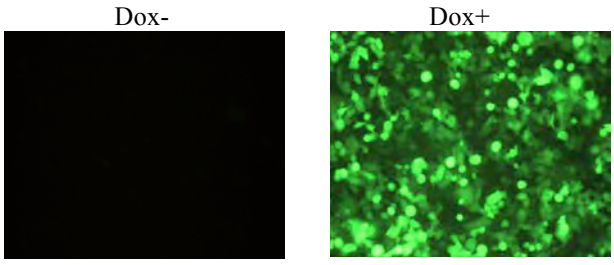


Fig.5 Doxycline induced EGFP expression in Hela cells transduced with a single adenoviral genome vector

3. Adeno-associated viral vector (AAV) system

The VVC is working on building the helper free AAV system. We have introduced this system from Stratagene. The helper virus free AAV system requires no replication competent virus detection and

can be easily worked on a BL-1 environment. We will work to construct a serial of replication defective AAV vectors for gene overexpression and gene knockdown in the following a couple of months.

Reportable outcomes

1. Two synthetic pathways for the synthesis of 3'-Deoxy-3'-[^{18}F]fluorothymidine using the NanoTek LF microfluidic synthesizer have been established.
2. Live imaging of C57Bl/6 mice injected with 10^6 B16/F10 murine melanoma cells using ^{18}FDG has been accomplished for the first time.
3. The VVC has been established.
4. The VVC has produced lentiviruses using the sh-RNA to autotaxin and a dominant negative STAT3 constructs.
5. An adenovirus-based doxycline-inducible gene expression and miRNA-mediated gene knockdown system has been developed.
6. The kockdown of ATX expression by lentivirus-mediated sh-RNA treatments has been validated in vitro.
7. Lentiviral delivery and stable expression of STAT3 constructs has been validated in vitro.
8. Work began on establishing AAV system.

Conclusion

During the first year of the award critical and formerly non-existing infrastructure has been created for the synthesis of a new PET tracer and the generation of recombinant viruses. In vitro proof of principle experiments validated the effectiveness of gene delivery using the viruses. In vivo proof of principle of tumor labeling and PET imaging using 3'-Deoxy-3'-[^{18}F]fluorothymidine has been achieved. These accomplishments position our team well toward the timely completion of the proposed studies during year 2.